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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
10 May 2002 (10.05.2002)

PCT

(10) International Publication Number  
**WO 02/36790 A2**

(51) International Patent Classification<sup>7</sup>: C12N 15/86, C07K 14/47, C12N 5/10, A61K 48/00, 39/00

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(21) International Application Number: PCT/EP01/12532

(22) International Filing Date: 30 October 2001 (30.10.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
00123687.6 30 October 2000 (30.10.2000) EP

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(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

#### Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: INFLUENZA VIRUS VECTOR FOR INFECTION OF DENDRITIC CELLS

(57) Abstract: The present invention relates to a method for the expression of tumor-associated antigens or virus associated antigens by patient derived immune cells such as by dendritic cells, patient derived immune cells obtainable by said method and their use for treating tumors or virus infections.

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## **Influenza Virus Vector for Infection of Dendritic Cells**

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The present invention relates to a method for preparing patient-derived immune cells, such as dendritic cells, capable of expressing antigens, such as tumor-associated antigens or virus associated antigens, patient derived immune cells obtainable by said method, their use for the treatment of tumors or virus  
10 infections and to a method for the expression of tumor-associated antigens or virus associated antigens by utilizing such patient-derived immune cells. The method utilizes attenuated, recombinant influenza virus vectors allowing efficient transduction and a high expression rate of genes encoding one or more tumor- or virus-associated antigens in patient-derived immune cells. The patient-derived  
15 immune cells obtained by said method can be used for ex-vivo therapy of cancer or diseases caused by viral microbial or parasitic infections..

### **Introduction**

Anti-tumor immune responses can be efficiently generated in vivo and in vitro by  
20 patient derived immune cells such as dendritic cells (hereinafter shortly referred to as "DC"), which have been genetically modified to express tumor-associated antigens (hereinafter shortly referred to as "TAA"). Several viral expression vectors derived from adenoviruses, retroviruses, and poxviruses have been used to transduce foreign genes into DC. These viruses contain DNA or form a DNA  
25 intermediate, which could integrate into the host genome with the risk of adverse mutations, and have limited host-cell range. In contrast, influenza viruses are sensitive to antiviral pharmaceuticals, fail to establish persistent infections, do not integrate their RNA genome into the chromosome, have a broad host-cell range, and may even be genetically designed entirely from  
30 cloned cDNA thus providing a favourable alternative. Here we show that self-attenuating recombinant avian influenza virus vectors very efficiently deliver TAA genes and other genes, which can include genes of virus associated antigens (hereinafter "VAA") into patient derived immune cells, in particular into human monocyte-derived DC. The modified DC retained their characteristic phenotypic  
35 and immunity-stimulating properties. Thus the influenza virus vector represents a valuable and safe tool in the development of immune cell- (DC-)based immunotherapy in man.

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DCs are highly potent initiators of immune responses and are widely distributed throughout lymphoid and non-lymphoid tissues (R.M. Steinman, *Fundamental Immunology*, fourth Ed. Ed W.E. Paul. Philadelphia: Lippincott-Raven. pp. 547 (1999)). To launch immune responses, DC have to capture and to process antigens in the periphery and to present them to rare antigen-specific T cells, which they encounter after migration to lymphoid organs. DC are therefore considered as "Nature's adjuvant" (J.W. Young, K. Inaba, *J. Exp. Med.* 183, 7-11 (1996); G. Schuler, R.M. Steinman, *J. Exp. Med.* 186, 1183-1187 (1997); J. Banchereau, R.M. Steinman, *Nature* 392, 245-252 (1998), M. Rescigno et al., *Immunol. Today* 20, 200-203 (1999)). Antigen uptake is accomplished by immature DC. They mature into potent T cell stimulators in response to environmental danger signals like pro-inflammatory cytokines, dsRNA or LPS. DC which are activated in this way, migrate to the lymphoid organs, develop cytoplasmic protruding veils and up-regulate a set of characteristic surface proteins. These are required for the interaction and activation of antigen-specific T cells. Among them are CD25, CD40, CD54, CD80, CD83, CD86 as well as high levels of MHC class I and class II molecules.

The ability to present antigens to naïve or quiescent T cells qualifies DC as a potent biological tool for cell-based immuno-therapeutical strategies directed against tumors and infectious diseases (G. Schuler, R.M. Steinman, *J. Exp. Med.* 186, 1183-1187 (1997); J. Banchereau, R.M. Steinman, *Nature* 392, 245-252 (1998)). Several studies reported beneficial anti-tumor and anti-viral effects due to DC mediated vaccinations in mice (J.I. Mayordomo et al., *Nat. Med.* 1, 1297-1302 (1995); R.C. Fields et al., *Proc. Natl. Acad. Sci.*, 95, 9482-9487 (1998); B. Ludewig et al., *J. Virol.* 72, 3812-3818 (1998)). Recently immunization with peptide- and/or tumor lysate-pulsed DC has also been reported in humans (F.J. Hsu et al., *Nat. Med.* 2, 52-58 (1996); G. Murphy et al., *Prostate* 29, 371-380 (1996); F.O. Nestle et al., *Nat. Med.* 4, 328-332 (1998); M.V. Dhodapkar et al., *J. Clin. Invest.* 104, 173-180 (1999); M. Marchand et al., *Int. J. Cancer* 80, 219-230 (1999); B. Thurner et al., *J. Exp. Med.* 190, 1669-1678 (1999)). Although these data are quite promising, the peptide-loading strategy has several disadvantages, including HLA restrictions and the fact that only a limited number of tumor-specific peptides are known.

On the other hand it is known that the strategy to genetically modify DC by genes coding for full-size tumor antigens has several advantages over pulsing DC with whole tumor lysates or tumor peptides: The heterologous proteins expressed from the introduced genes are endogenously processed, loaded onto MHC class I molecules (Y. Yang et al., *J. Virol.* 69, 2004-2015 (1995); C.A. Mack et al., *Hum. Gene Ther.* 8, 99-109, (1997)) and presented to T lymphocytes. Thus genetically modified DC have the potential to present previously unknown epitopes in association with different, individually divergent, MHC molecules. Therefore, new strategies for effective genetic modifications are needed in order to generate new DC based anti-tumor vaccines. DC can be generated in vitro from cultured human monocytes (N. Romani et al., *J. Exp. Med.* 180, 83-93 (1994); F. Sallusto, A. Lanzavecchia, *J. Exp. Med.* 179, 1109-1118 (1994); A. Bender et al., *J. Immunol. Methods* 196, 121-135 (1996); N. Romani et al., *J. Immunol. Methods* 196, 137-151 (1996)) even in large numbers from leukapheresis products for clinical use (B. Thurner et al., *J. Exp. Med.* 190, 1669-1678 (1999)). Transduction of these DC with tumor-associated or viral antigens leads to the presentation of these antigens via MHC class I molecules.

Viral vectors represent a very powerful method to deliver heterologous genes into primary cells. Several viral vector systems have been described that are capable to introduce foreign genes into DC. Adenoviral vectors have been reported to efficiently transduce human CD34<sup>+</sup>-derived (M. Br gni et al., *Gene Ther.* 5, 465-472 (1998)) as well as monocyte-derived DC (A.B. Dietz, P.S. Vuk, *Blood* 91, 392-398 (1998); L. Zhong et al., *Eur. J. Immunol.* 29, 964-972 (1999)). Retrovirus vectors have been shown to transduce proliferating human DC progenitors (J.F. Arthur et al., *Cancer Gene Ther.* 4, 17-25 (1997); P. Szabolcs et al., *Blood* 90, 2160-2167 (1997); B. Verhasselt et al., *Blood* 91, 431-440 (1998)). However, there are several disadvantages associated with retroviral vectors. They have relatively low transduction rates (P. Szabolcs et al., *Blood* 90, 2160-2167 (1997)) and the genomes of these vectors integrate into the host genome. Furthermore, non-dividing cells, like terminally differentiated, mature DC cannot be transduced. Vaccinia viruses have also been used to efficiently transfer genes into human DC. However this vector employs multiple mechanisms to evade the immune system including down modulation of the costimulatory molecule CD80 and induces apoptotic cell death in both stages of

DC (J. Engelmayer et al., J. Immunol 163, 6762-6768 (1999), R. Drillien et al., Virology 268, 471-481 (2000)).

Again, viral expression vectors derived from adenoviruses, retroviruses, and poxviruses have been reported to efficiently transduce foreign genes into DC (M.E. Reeves et al., Cancer Res. 56, 5672-5677 (1996); W. Song et al., J. Exp. Med. 186, 1247-1256 (1997); M. Di Nicola et al., Cancer Gene Ther. 5, 350-356 (1998); M. Subklewe et al, Blood 94, 1372-1381 (1999); L. Zhong et al., Eur. J. Immunol. 29, 964-972 (1999); S. Yang et al., J. Immunol. 164, 4204-4211 (2000)). These viruses contain DNA or have an DNA replication intermediate, which could integrate into the host chromosomal sequences thus posing the risk of adverse mutations. This is impossible with negative-strand RNA viruses as influenza viruses. Interestingly, DC infected by influenza viruses are strong stimulators of human CD8<sup>+</sup> T cells (N. Bhardwaj et al., J. Clin. Invest. 94, 797-807 (1994); A. Bender et al., J. Exp. Med. 182, 1663-1671 (1995) and Immunology 198, 552-567 (1998)). Furthermore, infection by influenza virus results in the maturation of immature DC and to increased antigen presentation and T cell stimulatory capacity (M. Cella et al., J. Exp. Med. 189, 821-829 (1999)). Therefore, gene transfer into DC by influenza virus vectors as disclosed in Strobel et al., J. of Dermatological Science 16(1), 5133 (1988) and J. of Investigative Dermatology 110(4), 605 (1998) was a promising approach to induce anti-tumor or anti-viral immune response.

The generation of recombinant Influenza viruses was hampered for a long time by the fact that the virus has a segmented RNA genome. The development of the RNA polymerase I technique allows the generation of recombinant viruses with additional genomic segments capable of expressing complete heterologous genes (G. Neumann et al., Virology 202, 477-479 (1994)), which was built around *in vivo* synthesis of recombinant vRNA molecules by cellular RNA polymerase I transcription of the respective template cDNA constructs. Modified terminal viral RNA sequences (hereinafter "promoter-up mutations" or promoter-up variants") were designed by nucleotide substitutions (Neumann and Hobom, Mutational analysis of influenza virus promoter elements *in vivo*, J. Gen. Virol. 76, 1709-1717 (1995); WO 96/10641). The above promoter-up variants carry up to five nucleotide substitutions (in promoter-up variant 1920; see Flick and Hobom, J. Gen. Virol. 80, 2565-2572 (1999)). When these promoter-up variants are

attached to a recombinant ninth vRNA segment its increased transcription and amplification rate will not only compensate for the losses suffered spontaneously, but even cause accumulation of the foreign vRNA segment during simple viral passaging, in the absence of any selection.

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However, due to its over-replication relative to all of the regular influenza vRNA segments (which of course are connected to wild-type promoter sequences) after catching up with the others the foreign segment will become over-abundant. This increasingly will result in viral particles that have incorporated several copies of recombinant vRNA, but no longer have a full set of all eight standard segments incorporated among an average of about 15 vRNA molecules present within a virion. Such particles are defective and will not result in plaque formation, hence after an initial increase of recombinant viral particles during the first steps of propagation a dramatic decrease is observed, usually at the third or fourth step of viral passaging, depending on the size of the recombinant vRNA and the level of the promoter-up mutation attached. A balanced situation with regard to the insert length and the level of promoter activity can be achieved, and has been propagated in a particular case over 11 passages, with essentially stable levels of recombinant viruses among a majority of helper viruses (around 80%) during these steps.

WO 00/53786 then discloses a recombinant influenza virus for high-yield expression of incorporated foreign gene(s), which is genetically stable in the absence of any helper virus and which comprises at least one viral RNA segment being an ambisense RNA molecule (ambisense RNA segment). Said ambisense RNA segment contains one of the standard viral genes in sense orientation and a foreign, recombinant gene in anti-sense orientation, or *vice versa*, in overall convergent arrangement. The ambisense RNA segment in the recombinant influenza virus of WO 00/53786 is - If stably integrated - one of the eight segments of the virus, and is a so-called "bicistronic" segment containing two different genes in covalent junction which are to be expressed by the recombinant virus. A further stable recombinant influenza virus with a bicistronic segment is described in PCT/EP01/08124. Here, the influenza virus comprises at least one viral RNA segment being a bicistronic RNA molecule coding for two genes in tandem arrangement (tandem RNA segment). In said tandem RNA segment one of the standard viral genes is in covalent junction with a foreign,

recombinant gene and said tandem RNA segment has an upstream splice donor and a downstream splice acceptor signal surrounding the proximal coding region. Both applications mention that such stable recombinant influenza viruses containing a foreign gene in a bicistronic segment are suitable as viral vectors for infection, transfection or transduction of patient-derived immune cells such as dendritic cells. These stable recombinant viruses are suitable for DC-based therapy when very pure, mature DC preparations are used, which afford abortive infections, but may be less desirable when DC preparations still contain some immature DC or monocytes. In the latter instance the use of attenuated recombinant viruses or self-attenuating recombinant viruses is preferred. The limited replication of self-attenuating viruses may in addition strengthen the immune response.

### Summary of the Invention

It was found that a recombinant influenza virus as disclosed in WO 96/10641 having a ninth, i.e. an additional, segment carrying a TAA or VAA gene under the control of a high-capacity promoter is a suitable vector for transduction of TAA and VAA genes. These viruses still abortively infect DC without interfering with their antigen presenting capacity but - due to their "instability" - are self-attenuating whilst retaining the high and persistent antigen expression controlled by the high-capacity promoter. The attenuated recombinant influenza viruses retain the immunostimulatory capacity of mature dendritic cells while expressing the antigens. In contrast to other viral vectors used for DC transduction - which often decrease DC function and have a limited host-cell range - influenza viruses can be efficiently controlled by antiviral pharmaceuticals, cannot integrate into host chromosomes and fail to establish persistent infections. The invention thus provides

(1) a method for preparing patient derived immune cells capable of expression of one or more tumor-associated antigens (TAA) or virus-associated antigens (VAA), said method comprising

(a) preparing a recombinant influenza virus having at least one additional segment containing

(i) a nucleotide sequence coding for the one or more TAA or VAA, and

(ii) terminal viral RNA sequences which have been modified by nucleotide substitutions in up to five positions, resulting in improved transcription

rates of both the vRNA promoter as well as the cRNA promoter as present in the complementary sequences; and

(b) infecting patient derived immune cells with the recombinant influenza virus obtained in step (a);

5 (2) a method for preparing patient derived immune cells capable of expression of one or more tumor-associated antigens (TAA) or virus-associated antigens (VAA), said method comprising

(a) preparing an attenuated recombinant influenza virus containing a nucleotide sequence coding for the TAA or VAA, wherein said nucleotide sequence is  
10 incorporated into the influenza virus genome resulting in an attenuated phenotype; and

(b) infecting patient derived immune cells with the recombinant influenza virus obtained in step (a);

(3) patient derived immune cells, preferably dendritic cells, expressing one or  
15 more TAA or VAA which are obtainable according to the method of (1) or (2) above;

(4) a pharmaceutical composition comprising patient derived immune cells of (2) above or a mixture thereof;

(5) the use of patient derived immune cells of (3) above for preparing a  
20 medicament for tumor therapy or antiviral therapy;

(6) a method for the expression of tumor-associated antigens or virus associated antigens in patient-derived immune cells which comprises utilizing (culturing) patient-derived immune  
cells of (3) above; and

(7) a method for treating tumors or virus infections in a mammal comprising administering  
25 the mammal patient derived immune cells of (3) above.

In the recombinant influenza virus used for infecting the patient derived immune cells of the method of embodiment (1), the additional gene incorporated into the influenza virus genome results in an attenuated phenotype. This is for example  
30 achieved by having the gene on an additional segment. However, other or additional mechanisms of viral attenuation may also be used and may lead to an attenuated or self-attenuating recombinant influenza virus vector (embodiment (2)).

35 Vaccination with dendritic cells presenting tumour antigens will induce a potent primary immune response or amplify existing cytotoxic antitumor T cell

responses. Therefore, tumor antigens most suitable for immunotherapy are, ideally, strictly tumor specific, or at least the immune response should have tumoral specificity. Many tumor antigens, however, are shared with normal cells and are overexpressed in the tumor. This implies that an immune response could potentially be harmful, if an immune response to self-antigens occurs causing autoimmunity. The technology for DC vaccines shall thus result in an immune response of sufficient quality and magnitude of tumor-specific T cell responses.

Tumor-specific antigens are rare. However, a growing family of testicular antigens has been identified that are aberrantly expressed in a significant proportion of tumors of various histological types and -in addition- only in testis cells. These antigens, called cancer/testis antigens, should ensure strict tumor specificity of the immune responses as the germ line cells do not express MHC-I molecules.

These antigens, for example the MAGE-, GAGE- and BAGE-families, NY-ESO-I or HOM-MEL-40 (a.k.a. SSX-2) are thus prime candidates for the DC-based anti-tumor vaccines when part of a potent dendritic cell vaccine based on influenza virus-mediated gene transfer. These antigens have the required selectivity for a flu-vector based DC vaccine, can most likely be readily incorporated into the recombinant virus, and are able to induce cellular immune responses. Peptides derived from MAGE-A3 have been "pulsed onto" dendritic cells, by Schuler and coworkers and the vaccine was found to induce specific CTL. This study could serve as an historical reference.

Furthermore, the number of tumor antigens suitable for potent therapeutic vaccines is still limited and a search for novel tumor antigens, as well as viral antigens, seems warranted. The Influenza virus vector system is suitable for antigen discovery. Co-expression of additional antigens with known cancer/testis antigens in an attenuated recombinant virus is an important option for widening the vaccine spectrum.

In experiments it was shown that genetically modified DC, which express tumor-associated antigens can efficiently induce anti-tumor immunity and thus have a high potential as tools in cancer therapy. The gene delivery is most efficiently achieved by viral vectors. Genes encoding a melanoma derived TAA, such as

MAGE-3 or the green fluorescence protein (GFP) were introduced into a high-expression self-attenuating avian influenza virus vector. Monocyte-derived mature DC infected by these recombinants efficiently produced GFP or MAGE-3. More than 90 % of the infected DC express a transduced gene. Importantly, these transduced DC retained their characteristic phenotype, their potent allogeneic T cell stimulatory capacity and were able to stimulate MAGE-3 specific CD8<sup>+</sup> cytotoxic T cells. Thus influenza virus vectors provide a highly efficient gene delivery system to transduce human DC with TAA, which consequently stimulate TAA specific T cells, also when attenuated, for example, but not limited to, by expressing the TAA from a ninth, additional segment under the control of a high-capacity promoter.

### Description of the Figures

Fig.1: Insertion of the MAGE-3 gene into recombinant influenza viruses: A plasmid containing the MAGE-3 (or GFP) open reading frame flanked by influenza virus promoter-up variant signals and more externally RNA-polymerase I control sequences was transfected into 293T cells. These cells were infected with influenza viruses. The heterologous cDNA sequences are transcribed by the cellular RNA polymerase I in virus RNA- (vRNA-) like molecules, which are subsequently amplified by the viral polymerase. The heterologous vRNA and the eight homologous influenza virus genomic RNAs are all co-packaged into virus particles.

Fig. 2: High level expression of heterologous proteins from recombinant influenza viruses: MDCK cells were infected with recombinant influenza virus stocks expressing MAGE3-AU1 (FPV-MAGE3-AU1) or GFP (FPV-GFP) under control of promoter-up variant 1104.

(A) Heterologous RNA produced by the recombinant viruses was detected by RT-PCR with primers specific for MAGE-3 and GFP, respectively. The  $\beta$ -actin RT-PCR served as an internal control. The PCR products were detected in ethidium bromide stained agarose gels.

(B) Proteins extracted from infected cells were analyzed by immunoblot using specific antibodies directed against the AU-1 sequence tag of MAGE3-AU1(left panel). The corresponding bands were visualized by a secondary antibody conjugated with horseradish peroxidase and an enhanced chemoluminescence (ECL) assay. The same blot was reprobated using antibodies against GFP (right panel).

Fig. 3: Efficient gene transfer by recombinant influenza virus into human DC: Mature human dendritic cells were infected with the recombinant influenza virus FPV-GFP at a MOI = 1.

(A) After 8 hours cells expressing GFP were detected by UV-fluorescence microscopy (right panel). The left panel shows the same detail in phase contrast microscopy.

(B) Quantification of the transduction rate by FACS analysis: More than 90% of the cells were found to express GFP. Uninfected and FPV wildtype infected DC were used as controls.

Fig. 4: Expression of heterologous MAGE-3 protein from recombinant influenza virus transduced human DC: Mature human DC were infected (MoI = 1) with the influenza virus recombinants FPV-MAGE3-AU1 or FPV-GFP. After 24 hours, proteins were extracted, separated and analyzed by the immunoblot technique.

(A) To detect the MAGE3-AU1 fusion protein the blot was incubated with specific antibodies to the AU-1 sequence tag. The corresponding band was visualized by a secondary antibody conjugated with horseradish peroxidase and an enhanced chemiluminescence (ECL) assay.

(B) To detect GFP the same blot was reprobed using specific antibodies.

Fig. 5: Unchanged DC surface marker expression after gene transfer by

recombinant influenza virus: Mature DC were infected (MoI = 1) with FPV-GFP or left uninfected. After 24h, cells were counter-stained using antibodies directed CD86, CD80, HLA class I and class II and secondary PE-conjugated goat anti-mouse IgG F(ab')<sub>2</sub> fragments. The expression of cell surface markers and GFP was quantified by flow cytometry. Percentages of cells in the quadrants are indicated.

Fig. 6: Influenza virus transduced DC are potent T cell stimulators: The allo-stimulatory capacity of DC infected with FPV-GFP(●), with FPV-MAGE3-AU1(◆) or with the FPV wild type strain(▲) was tested in a MLR assay. As indicated, graded DC (6000-220) were co-cultivated with  $2 \times 10^5$  T cells. After four days, cells were pulsed with [<sup>3</sup>H]-thymidine for 16 hours and incorporation of radioactivity was determined. Shown are the mean counts ± SEM of triplicates. Counts for T cells alone or DC alone were always less than 1000 cpm.

Fig.7: Detection of MHC-I/IMP complexes on the transduced DC by a specific CTL clone: Uninfected or FPV-MAGE3-AU1 infected DC ( $7.5 \times 10^4$ ) were co-cultured with an IMP-specific CTL clone ( $5 \times 10^3$ ) for 18 hours. The capacity of the CTL clone to release IFN- $\gamma$  was immunochemically visualized by the ELISPOT-technique. Results are shown as mean spot numbers  $\pm$  SEM of triplicates.

Fig. 8: Expansion of CD8<sup>+</sup> memory T cells in the absence of exogenous cytokines by transduced DC: To demonstrate that DC infected with recombinant influenza virus are able to stimulate influenza virus-specific CTL, autologous T cells ( $4 \times 10^6$ ) were co-cultured with  $0.2 \times 10^6$  DC for seven days without addition of exogenous cytokines.

(A) Subsequently the IMP-specific CTLs were stained with tetrameric MHC-I/IMP complexes at 37°C and counterstained with labelled CD8 mAb at 4°C. The IMP-specific CD8<sup>+</sup> T cells were identified by FACS analyses (upper right sector). The dot plots show T cells co-cultured with uninfected DC (control), IMP peptide-pulsed DC (IMP-peptide), FPV-GFP infected DC or with FPV-MAGE3-AU1 infected DC.

(B) Demonstration of IFN- $\gamma$  release from IMP-specific CTLs. The CTL activity upon stimulation with IMP peptide (1 $\mu$ M) was assessed by ELISPOT-assay with  $0.5 \times 10^6$  T cells per well. Cells were cultured for 18 hours and IFN- $\gamma$  release from individual T cells was immunochemically visualized. Shown are means  $\pm$  SEM of triplicates

Fig. 9: Stimulation of MAGE-3 specific CTL by transduced DC: Uninfected or FPV-MAGE3-AU1 infected DC ( $7.5 \times 10^4$ ), were co-cultured with MAGE-3 specific CTL clone ( $5 \times 10^3$ ) for 18 hours. The capacity of transduced DC to present antigen was evaluated by IFN- $\gamma$  secretion of individual CTL using the ELISPOT-technique. Results are the mean of three experiments  $\pm$  SEM.

### **Detailed Description of the Invention**

"Patient derived Immune cells" according to the present invention comprises antigen presenting cells including, but not limited to, dendritic cells. The patient derived immune cells are derived from mammals, preferably humans.

The provision of patient derived immune cells utilized in the method of embodiments (1) and (2) is hereinafter described in more detail with reference to DC (again, the present invention is not limited to the use of DC). The (human) DC can be derived from various sources including peripheral blood, bone marrow

etc. The DC generation starting peripheral blood mononuclear cells (CPBMC) is preferred (see WO 93/20185, WO 97/29182, EP-A-922 759, incorporated herewith in its entirety). PBMC can be isolated from buffy coats by Ficoll-Hypaque density gradient centrifugation. CD14 cells are then selected (e.g. by using electric microbeads) from the PBMC, cultured and differentiated in a suitable medium (e.g. RPMI supplemented with plasma and IL-4) for about 1 to 6 days, and matured by adding a suitable maturation cocktail (e.g. containing IL-1 $\beta$ , IL-6, TNF- $\alpha$  and PGE<sub>2</sub> to the culture medium and further culturing for about 1 to 4 days. After washing with serum-free medium (e.g. RPMI) the mature DC are ready for infection).

Infection of the DC according to step (b) of embodiment (1) and (2) of the invention is achieved by adding recombinant virus vector (at a MoI of about 0.1 to 5, preferably about 1) to a DC culture in serum-free medium (e.g. RPMI or the like) for about 0.5 to 5 h (preferably at 37 °C/5% CO<sub>2</sub>). Thereafter the infected DC are washed with medium (RPMI or the like) and are cultured in medium supplemented with plasma (e.g. RPMI + 1% AB-plasma).

The recombinant influenza viruses used in embodiment (1) preferably contain a ninth segment which is a so-called "monocystronic" segment (viz, it contains one TAA or VAA gene flanked by the terminal viral RNA sequences).

Moreover it is preferred in embodiments (1) and (2) that the terminal viral RNA sequences, which are active as promoter signal, are modified by nucleotide substitution in up to 5 positions, resulting in improved transcription rates (of both the vRNA promoter and the cRNA promoter as present in the complementary sequence) as well as enhanced replication and/or expression rates relative to the wild-type sequence. Said modified terminal viral RNA sequences preferably differ from the wild-type sequence in that in said ninth vRNA segment the 12 nucleotide conserved influenza 3' terminal sequence has been modified by replacement of one to three nucleotides occurring in said sequence at positions 3, 5 and 8 relative to the 3' end by other nucleotides provided that the nucleotides introduced in positions 3 and 8 are forming a base pair (i.e., if the nucleotide position 3 is G, than that in position 8 is C; if the nucleotide in position 3 is C, than that in position 8 is G; etc.).

The 3' conserved regions of the wild-type influenza virus have the following sequences:

Influenza A: (5')-CCUGCUUUUGCU-3'

Influenza B: (5')-NN(C/U)GCUUCUGCU-3'

5 Influenza C: (5')-CCUGCUUCUGCU-3'.

Moreover, the 13 nucleotide conserved influenza 5'-terminal sequence may be modified by replacement of one or two nucleotides occurring in said sequence as positions 3 and 8 by other nucleotides, again provided that the introduced nucleotides are forming a base pair. The 5' conserved regions of the wild-type

10 influenza virus have the following sequences:

Influenza A: 5'-AGUAGAAACAAGG

Influenza B: 5'-AGUAG(A/U)AACA(A/G)NN

Influenza C: 5'-AGCAGUAGCAAG(G/A):

15 Preferred influenza viruses of the invention are those wherein in the 3' conserved region the replacements G3A and C8U have been performed, more preferred are those where also the replacement U5C has been performed (the above mutations are annotated relative to the 3' end; such counting from the 3' end is also indicated by a line on top of the digit, e.g., G  $\bar{3}$ A). Another preferred  
20 influenza virus mutant comprises the 3'-terminal nucleotide sequence G3C, U5C and C8G (relative to the 3' end) resulting in the following 3' terminal nucleotide sequence (5')-CCUGGUUCUCCU-3'. Among the influenza viruses defined hereinbefore those having a 3'-terminal nucleotide sequence of (5')-CCUGUUUCUACU-3' are most preferred. In case of an influenza A virus the  
25 segment may further have the modifications U3A and A8U in its 5' terminal sequence, in case of influenza C it may have the modifications C3U and G8A in its 5' terminal sequence. The most preferred influenza viruses of the present invention comprise the following general structures:

Influenza A (mutant pHL1102):

30 5'-AGUAGAAACAAGGNNNU<sub>5-6</sub>..(880-2300 ntds)..N'N'N'CCUGUUUUUACU-3'

Influenza A (mutant pHL1104):

5'-AGUAGAAACAAGGNNNU<sub>5-6</sub>..(880-2300 ntds)..N'N'N'CCUGUUUCUACU-3'

Influenza A (mutant pHL1920):

5'-AGAAGAAUCAAGGNNNU<sub>5-6</sub>..(880-2300 ntds)..N'N'N'CCUGUUUCUACU-3'

35 Influenza A (mutant pHL1948):

5'-AGUAGAAACAAGGNNNU<sub>5-6</sub>..(880-2300 ntds)..N'N'N'CCUGGUUCUCCU-3'

Influenza B:

5'-AGUAG(A/U)AACA(A/G)NNNNNU<sub>5-6</sub>..(880-2300 ntds)..N'N'N'N'(C/U)GUUUCUACU-3'

Influenza C:

5'-AGUAGUAACAAG(G/A)GU<sub>5-6</sub>..(880-2300 ntds)..CCCCUGUUUCUACU-3'

- 5 In the above structures the variables are defined as follows:
- (1) Underlined and enlarged letters show the required mutations relative to the wild-type sequence for preparing a promoter mutant with enhanced properties;
  - (2) enlarged A in position 10 in the 5'-part of the sequence: unpaired A residue, bulge-forming;
  - 10 (3) (A/G) in one position: different isolates or single segments with variable sequence at the respective position, which are functionally interchangeable;
  - (4) N and N': positions undefined, but base-paired relative to each other because of complementarity between the 5' and 3' termini, different among the 8 segments, but constant for each segment throughout all viral isolates;
  - 15 (5) (880-2300 ntds): the lengths of the viral RNA segments, in case of segments with foreign genes increased up to 4,000 nucleotides.

Here is described a new transduction system for DC based on a recombinant avian influenza virus vector. Using this vector more than 90 % of the DC

20 expressed the marker green fluorescent protein (GFP) and similarly the tumor-associated antigen MAGE-3 was efficiently expressed. These transduced DC retained their characteristic phenotype and their potent T cell stimulatory capacity. Furthermore, they were also able to stimulate MAGE-3 specific CD8<sup>+</sup> memory T cells as well as CTL clones. As this recombinant virus is an attenuated

25 virus, other attenuated recombinant influenza viruses that show limited or no replication in vivo, but do allow expression of foreign genes will be equally efficacious. Such recombinant viruses can be obtained by deleting genes not required for replication in vitro, or by deleting genes and supplementing these genes in trans in producer cell lines, as known to those skilled in the art.

30

Expression of heterologous genes in dendritic cells (DC) is a powerful strategy to elicit strong immune reactions against tumor antigens. Here we report that influenza viruses are capable to efficiently introduce and express foreign proteins such as tumor associated antigens (TAA) like MAGE-3 or virus associated

35 antigens (VAA) in functional monocyte-derived human DC, thus providing a potential tool for immune therapy of malignant melanoma and other tumors.

Suitable TAAs in accordance with the present invention are those known in the art, including, but not limited to, TAAs from the class of cancer/testis antigens. Preferably the TAA is MAGE-3, NY-ESO-1 or HOM-MEL-40.

- 5 Suitable VAAs in accordance with the present invention are those known in the art including, but not limited to, VAAs selected from a Herpes simplex virus immediate early or early protein, a human papilloma virus-antigen, preferably L<sub>1</sub>, E<sub>5</sub> or E<sub>6</sub>, a Human Immunodeficiency Virus protein, preferably tat, rev, nef or gag, or a Hepatitis C virus protein known to elicit T cell response in a mammal.
- 10 The TAA or VAA may also be a fusion protein, preferably a fusion protein comprising entirely or partially the TAA or VAA proteins listed above.

In experiments it was shown that more than 90 % of mature DC expressed the GFP marker after infection with recombinant influenza virus without undergoing

15 lysis. This is comparable with the very high transduction rate reported for recombinant GFP adenoviral vectors (L. Zhong et al., Eur. J. Immunol. 29, 964-972 (1999)). However adenovirus has no receptor on the DC and very high multiplicities of infection are needed to transduce genes into DC, which can lead to immunosuppression. The DC transfected with the specific recombinant

20 influenza viral vector did not reveal changes in their typical surface marker expression with the exception of a moderate down-modulation of CD83. This is most probably a strain specific feature of FPV, since infection with another influenza virus strain (PR-8) had no impact on the typical surface expression of this protein (M. Cella et al., J. Exp. Med. 189, 821-829 (1999)). The DC

25 transfected with the recombinant Influenza virus vector express viral and heterologous protein and generate MHC-I peptide complexes as shown by using CTL clones in conjunction with Interferon- $\gamma$  ELISPOT. The immunogenicity of the transfected DC was also shown. They were able to expand influenza virus matrix protein-specific memory T cells in the absence of exogenous cytokines. This is a

30 unique feature of mature potent Immunostimulatory DC. Furthermore, infected DC were still very potent allogeneic T cell stimulators and were also able to stimulate MAGE-3 specific CTL clones. Therefore, mature DC which have been subjected to influenza vector-mediated heterologous gene transfer are potent antigen presenting cells.

35

The features of this influenza virus vector suggest that it can be developed into a safe vector system useful also for the application in humans, e.g. for a *ex vivo* tumor or antiviral therapy. In contrast to other viral vector systems, influenza virus does not contain DNA nor does its replication depend on a DNA intermediate. Thus influenza virus sequences cannot integrate into genomic host sequences and cause mutations or cancer. There is no clinical indication that influenza virus infection can become persistent or chronic in humans, as has been known for retroviruses and adenoviruses (H. Hayder A. Mullbacher, Immunol. Cell. Biol. 74, 504-512 (1996)).

Recombinant influenza viruses utilized in embodiment (1) of the current invention have a limited capacity to replicate in culture, thus can be considered as self-attenuating. Usually after four passages the infectivity of viral stock is drastically decreased. The self-attenuation is due to the superior capacity of the promoter variant driving expression of the gene(s) on the heterologous segment. Thereby it prevents the incorporation of essential genomic segments into viral particles after exceeding a certain threshold. Finally, several antiviral substances including neuraminidase inhibitors are known to interfere with any unwanted influenza virus, propagation providing a backup in case of an accidentally induced infection (R. Lambkin, J.S. Oxford, Textbook on Influenza, p. 487 (1998); C.R. Penn, Textbook on Influenza, pp. 477-487 (1998)).

Like other viral vector systems used for gene transfer into DC, the influenza virus vector results in the presentation of viral vector antigens in addition to the TAA. Simultaneous presentation of several antigens could result in unequal immune reactions with the activation of CTL against one or few dominant antigens. Such immunodominance in the CTL response probably results from interference between T cell responses and not from insufficient presentation of peptide epitopes (E.Z. Wolpert et al., J. Immunol. 161, 4449-4505 (1998)). Immunodominance of viral vector antigens over heterologous tumor associated antigens cannot be excluded for all MHC-I haplotypes. However, the activation of a MAGE-3-specific CTL clone by influenza virus-transduced DC strongly argues for effective presentation of the TAA. Furthermore, since dominant T cell epitopes for influenza viruses are well characterized and influenza viruses can be generated entirely from cloned DNA (G. Neumann et al., Proc. Natl. Acad. Sci., 96, 9345-9350 (1999)), competing viral epitopes in the vector may be removed

by mutagenesis, if necessary. For long-term genetic modifications, the immunogenicity of the used vector might be a major obstacle, since the cellular immunity directed against influenza virus could lead to the destruction of genetically modified DC. This has been reported for instance for adenoviral  
5 vectors upon application of these vectors to elicit anti-tumor immune responses in gene therapy studies (G.P. Gao et al., J. Virol. 70, 8934-8943 (1996) and Immunology 91, 135-144 (1997); M. Christ et al., Immunol. Lett. 57, 19-25 (1997)). On the other hand, for immunotherapy studies, using short-lived genetically modified DC this cellular immunity problem might not be as severe.

10 The presence or development of neutralizing antibodies against the viral vector might not be of major concern in an ex vivo gene transfer approach with no production of viral progeny. Influenza viruses are internalized upon DC infection, which also becomes abortive during late gene expression (A. Bender et al., Exp. Med. 182, 1663-1671 (1995) and Immunobiology 198, 552-567 (1998)). This  
15 suggests that the chances of direct exposure of complete viral surface antigens that can be recognized by host antibodies are reduced. Additional techniques to attenuate the virus are available leading to further reduction in replication (Hobom, unpublished). Finally, since it is now possible to efficiently generate influenza A viruses entirely from cloned cDNAs (Neumann et al., 1999) it is  
20 feasible to directly introduce new haemagglutinin genes into the viral vector which are not recognized by the immune system. In addition, to using influenza virus vectors based on hemagglutinins from different influenza serotypes, these vectors might also be applied in combination with other viral vectors in a sequential approach to boost the immune reaction against the heterologous  
25 antigens and to minimize the reaction to the viral proteins.

The pharmaceutical composition according to embodiment (4) above and the medicament according to (5) above are suitable in ex vivo and in vivo application schemes contain the recombinant influenza virus in a pharmaceutically effective  
30 amount. Besides said recombinant influenza virus, the pharmaceutical composition and the medicament may contain further pharmaceutically acceptable carrier substances well-known to a person skilled in the art, such as binders, desintegrants, diluents, buffers, preservatives, etc. The pharmaceutical composition and medicaments are solid or liquid preparations and are suitable to  
35 be administered orally, intravenously or subcutaneously.

The pharmaceutical composition and medicament are suitable as (*ex vivo*) vaccines. The method of embodiment (7) of the invention includes the administration of an effective amount to the mammal or the administration of a sufficient infective dose of the recombinant virus to the cell system that is used for *ex vivo* therapy or for *in vitro* investigations, whereby the amount and dose will be determined by a person skilled in the respective arts or knowledgeable of the desired treatments. Said method is an autologous immunotherapy wherein the patient derived cells which are *ex vivo* infected, and the transduced cells are reintroduced into the patient.

The method according to embodiment (6) of the invention pertains to the expression of the VAA or TAA by the patient derived immune cells obtained in step (b) of embodiments (1) and (2).

In summary, the influenza virus expression vector system offers a unique combination of features which are advantageous for an *ex vivo* transduction of DC. It combines high efficiency with genomic simplicity, lack of genomic integration, and the availability of a chemotherapeutic backup safety measure. Thus influenza virus vectors may be a valuable alternative in the development of genetically modified DC. To minimize the anti-vector immunization and booster the therapeutic anti-tumor immune responses, different influenza viral vectors based on different strains can be used in sequential immunization protocols. The invention is further explained by the following non-limitative experiments.

## Experiments

1. Materials and Methods, Cell lines and viruses: The human embryonic kidney cell line 293T and the Madin-Darby canine kidney (MDCK) cell line were cultivated in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), glutamine (0.35 mg/ml), penicillin (0.12 mg/ml) and streptomycin sulfate (0.12 mg/ml). The medium for 293T cells was additionally supplemented with G418 (500 µg/ml) in order to maintain high T-Ag expression.

The human melanoma cell line MZ2-MEL 43, the EBV-transformed B cell line LG-2-EBV and the HLA-A1 restricted MAGE-3-specific CTL clone LB 705 CTL 434/1 were kindly provided by P. v.d. Bruggen (Ludwig Institute for Cancer Research;

Brussels, Belgium). MZ2-MEL 43 and LG-2-EBV cells were cultured in DMEM or Iscove's medium (Life Technologies, Karlsruhe, Germany) supplemented with 10% FCS. The MAGE-3.A1 CTL clone was cultured in Iscove's medium supplemented with 10 % pooled human serum, L-arginine (116 µg/ml), L-asparagine (36 µg/ml) and gentamycin (10 µg/ml). This CTL clone was restimulated weekly with irradiated (100 Gy) MAGE-3.A1 peptide loaded MZ2-MEL 43 cells. Irradiated (100 Gy) LG-2-EBV cells were used as feeder cells. The CTL clone C9, which specifically recognizes the HLA-A2.1 restricted epitope of influenza virus matrix protein 1 (IMP) was cultured in the same medium as the MAGE-3.A1 CTL clone. The CTL clone C9 was re-stimulated with PHA-treated irradiated (30 Gy) PBMC as stimulator cells. Again, irradiated (100 Gy) LG-2-EBV were used as feeder cells. All cell lines and clones were maintained at 37°C in an 8 % CO<sub>2</sub> atmosphere.

The avian Influenza A virus (fowl plaque virus, FPV H7N7) was propagated in MDCK cells as previously described (G. Neumann, G. Hoborn, J. Gen. Virol. 76, 1709-1717 (1995)). Infection at a multiplicity of infection (MoI) of  $1 \times 10^{-5}$  to  $1 \times 10^{-6}$  resulted in virus stocks containing approximately  $1 \times 10^9$  plaque forming units (pfu) per ml after two days of incubation. As a measure of the virus particle content, titers of virus hemagglutination were determined using chicken erythrocytes. For the quantification of infectious particles, plaque assays and limiting dilution assays were performed on MDCK cells as described previously (A. Bender et al., J. Exp. Med. 182, 1663-1671 (1995)). To determine the fraction of viruses expressing the heterologous green fluorescent protein (GFP), limiting dilution assays were examined using a fluorescence microscope (Leica DM IRB; Leica, Bensheim, Germany).

2. Preparation of recombinant Influenza virus: Heterologous genes were introduced into Influenza viruses by the application of the RNA polymerase I technique (A. Zobel et al., Nucleic Acid Res. 21, 3607-3614 (1993)). This strategy leads to the incorporation of a heterologous gene into the influenza virus particle as an additional, ninth genomic segment (pseudo-vRNA). The required RNA is transcribed from transfected plasmid DNA, which encodes the recombinant gene in combination with modified influenza virus 3' plus 5' terminal sequences (Fig.1). These constitute an Influenza virus promoter-up variant and contain the viral promoter and packaging signals. For expression of the influenza

virus pseudo-vRNA the plasmid contains RNA polymerase I transcription control elements, that allow the generation of uncapped, non-polyadenylated RNA with the flanking influenza virus control sequences. Influenza virus recombinants were essentially prepared as described before (G. Neumann et al., Virology 202, 477-479 (1994)). Briefly, plasmids (3 µg) encoding the heterologous viral genomic segment with a promoter-up mutation (G. Neumann, G. Hobom, J. Gen. Virol. 76, 1709-1717 (1995)) were transfected into 293T cells cultured in 6cm plates using Lipofectamin Plus (Life Technologies, Karlsruhe, Germany). After 24 hours, medium was removed from the culture and cells were inoculated with 2 ml of virus suspension (FPV Bratislava) in phosphate buffered saline (PBS) supplemented with CaCl<sub>2</sub> (0.91mM) and MgCl<sub>2</sub> (0.49mM) (=PBS++) at a MoI of 2. After 45 min, the cells were washed once with PBS++ before 3 ml of medium were added. Eight hours later, after one replication cycle, the supernatant containing the recombinant virus was harvested. To enrich for recombinant viruses, stocks were serially passaged on MDCK cells at a MoI of 2 or 3. After three passages most viruses contained the heterologous sequence as was monitored by GFP expression in limiting dilution assays. The enrichment is due to the higher replication rate of the heterologous fragment compared to the wild type segments originating from the helper virus.

3. Plasmid construction: The plasmid pHH10 contains all *cis*-acting sequences required for the synthesis of an influenza virus genomic segment. These include the influenza virus vRNA 5' and 3' terminal sequences with promoter and packaging signal activity which are flanked by an RNA polymerase I (pol I) promoter and an RNA polymerase I terminator element. Heterologous sequences were cloned between the two parts of the influenza virus promoter in negative orientation relative to the pol I promoter, using the pBSK multiple cloning site present in this location. The coding region for MAGE-3 was generated by PCR amplification of a 983 bp fragment using plasmid pcDNA1-MAGE3 (B. Gaugler et al., J. Exp. Med. 179, 921-930 (1994)) as template (kindly provided by P. v. d. Bruggen, Brussels, Belgium). The sense primer adds a *Bgl* II site to the 5' end (5'- GGA GAT CTC ATC ATG CCT CTT GAG CAG -3') and the reverse primer adds an AU1 tag (P.S. Lim et al., J. Infect. Dis. 162, 1263-1269 (1990)) as well as an *Xba* I site to the 3' end (5'- CCT CTA GAT TAT ATA TAG CGA TAG GTG TCC TCT TCC CCC TCT CTC -3'). The PCR fragment was cleaved using *Bgl* II and *Xba* I, purified from agarose® gel (QIAEX II gel extraction kit, Qiagen, Hilden, Germany)

and cloned into pHH10 via *Bam* *HI* and *Xba* *I*. The resulting plasmid was designated pM4HH. The plasmid pHL2251 is a derivative of pHH10 containing a variant of the *Aequorea victoria* GFP gene.

5 4. RT-PCR: Total RNA was extracted from infected MDCK cells 8 hours post infection using the High Pure RNA Isolation Kit (Roche Molecular Biochemicals, Mannheim, Germany). Subsequently, RT-PCR was performed using the Titan One Tube RT-PCR System (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturers instructions. The PCR amplification was  
10 performed using the following conditions: 95°C for 2 min (first cycle only), 95°C for 20 s, 57°C for 20 s, 72°C for 45 s (21 cycles), 95°C for 20 s, 57°C for 20 s, 72°C for 60 s (19 cycles), 72°C for 4 min. Each RT-PCR reaction was performed in a final volume of 25 µl, with 1 µl of total RNA as template. The nucleotide sequences of the PCR primers used, were as follows: sense 5'-CGG GAA ATC  
15 GTG CGT GAC AT -3' and reverse 5'-GAA CTT TGG GGG ATG CTC GC -3' yielding a 712 bp beta-actin fragment; sense 5'- TAA TAC GAC TCA CTA TAG GGC -3' and reverse 5'- CAG CAC GTG TCT TGT AGT TCC -3' yielding a 397 bp GFP fragment; sense 5'- CAG CAC GTG TCT TGT AGT TCC -3' and reverse 5'-  
ATA TAG CGA TAG GTG TCC TC -3' yielding a 747 bp MAGE-3-AU1 fragment.

20 5. Immunoblot: Total cellular protein from infected MDCK cells and DC was prepared according to a freeze-thaw lysis protocol (I. Schmitt et al., J. Virol. 72, 633-640 (1998)). The lysis buffer contained 1 mM PMSF, 0.02mg/ml Aprotinin, 0.02 mg/ml Leupeptin and 2 mM DTT in 1 x TNE (Tris, NP-40, EDTA). After  
25 electrophoresis on a 12% polyacrylamide gel, proteins were transferred onto Immobilon P membrane (Millipore, Eschborn, Germany), and the blots were incubated with an anti-AU1 mouse monoclonal antibody (1:5000) (Hiss Diagnostics, Freiburg im Breisgau, Germany), washed three times and then incubated with a secondary anti-mouse antibody (1:2000) conjugated with  
30 horseradish peroxidase (Amersham Buchler, Braunschweig, Germany). GFP on immunoblots was detected with an anti-GFP rabbit polyclonal antibody (1:1000) (Clontech, Heidelberg, Germany) and a secondary anti-rabbit antibody (1:2000) also conjugated with horseradish peroxidase (Amersham Buchler, Braunschweig, Germany). Proteins recognized by these antibodies were detected using an  
35 enhanced chemoluminescence assay (Amersham Buchler, Braunschweig, Germany).

6. Generation and infection of human DC: Dendritic cells were generated from CD14<sup>+</sup> human monocytes. PBMC were isolated from buffy coats of healthy HLA-typed donors by Ficoll-Hypaque density gradient centrifugation (460 x g, 25 min, 20°C). To remove platelets, PBMC were repeatedly centrifuged with decreasing g numbers. CD14<sup>+</sup> cells were positively selected using magnetic microbeads (Miltenyi, Bergisch Gladbach, Germany). As assessed by FACS-analyses, the CD14<sup>+</sup> cells were on average over 95% pure. Immunophenotypically these CD14<sup>+</sup> cells were HLA-class I<sup>++</sup>, HLA-DR<sup>++</sup>, CD86<sup>++</sup>, CD40<sup>+</sup>, CD83<sup>-</sup>, CD80<sup>-</sup>, CD25<sup>-</sup>

. The isolated CD14<sup>+</sup> cells were cultured at a density of  $7 \times 10^6$  in a volume of 12 ml in 10 cm dishes (Falcon 3003, Becton Dickinson Heidelberg, Germany) in RPMI (BioWhittaker, Walkersville, MD, USA) supplemented with 1 % heat-inactivated AB-plasma. To start the differentiation to DC, 800 U/ml rhGM-CSF (Leukomax, Novartis, Basel, Switzerland) and 1000 U/ml rhIL-4 (Novartis Research Institute, Vienna, Austria) were added (day zero). On day three one third of the culture medium was replaced with fresh culture medium supplemented with 400 U/ml rhGM-CSF and 500 U/ml rhIL-4. On day six the final maturation of DC was induced by adding IL-1- $\beta$  (2 ng/ml) (Sigma, Deisenhofen, Germany), IL-6 (1000 U/ml) (Novartis, Basel, Switzerland), PGE<sub>2</sub> (1  $\mu$ g/ml) (Sigma) and TNF- $\alpha$  (10 ng/ml) (Boehringer Ingelheim Austria, Vienna, Austria) according to H. Jonuleit et al., Eur. J. Immunol. 27, 3135-3142 (1997). On day eight or nine FACS-analyses revealed the typical cell surface marker expression of mature DC: CD25<sup>++</sup>, CD40<sup>++</sup>, CD80<sup>++</sup>, CD83<sup>++</sup>, CD86<sup>++</sup>, HLA-DR<sup>++</sup>, HLA-class I<sup>++</sup>, CD14<sup>-</sup>. On day eight or nine mature DC ( $1 \times 10^6$ ) were infected in 1 ml serum-free medium for 1 hour at 37°C/5% CO<sub>2</sub> at a MoI of 1. Thereafter, cells were washed extensively with RPMI and cultured in 6-well-plates in RPMI supplemented with 1 % AB-plasma.

7. Immunophenotyping with antibodies and tetramers: Cell surface antigens were detected by incubating cells ( $1 \times 10^6$ / ml) for 30 min at 4°C with monoclonal antibodies specific for CD25 (clone 2A3) from Becton Dickinson (Heidelberg, Germany), CD14 (UCHM-1), CD40 (B-B20), CD86 (BU-63), HLA class I (W6/32), HLA-DR (DDII), all from Cymbus Biotechnology (Chandlers Ford Hants, U.K.), CD80 (MAB104), and CD83 (HB15a) both from Immunotech (Hamburg, Germany). After washing, cells were incubated with PE-conjugated goat anti-mouse IgG F(ab')<sub>2</sub> fragments (Jackson Immuno Research, West Grove,

PA, USA) for 30 min at 4°C. FACS-analyses were performed on a FACScan (Becton Dickinson, Heidelberg, Germany) using the CellQuest software. For the detection of HLA-A2.1/IMP-specific CTL, cells were incubated with PE-labelled tetrameric MHC-I/IMP peptide complexes (tetramers) (Dunbar *et al.*, 1998) for 15 min at 37°C. Subsequently cells were counterstained with Tricolor-labelled CD8 specific mAb (Caltag, Burlingame, CA, USA) for 15 min on ice. After several washes  $0.5 \times 10^6$  cells were analyzed by flow cytometry.

8. T cell stimulation assays: For mixed lymphocyte reaction (allo-MLR), allogeneic CD4<sup>+</sup> or CD8<sup>+</sup> T cells were isolated by positive magnetic cell sorting according to the manufactures instructions (Miltenyi, Bergisch Gladbach, Germany). The T cells ( $2 \times 10^5$ /well) were cocultured with various numbers (220, 660, 2000, 6000) of DC in 96-well flat bottomed plates. After 4 days, cells were pulsed with 1  $\mu$ Ci/well [<sup>3</sup>H]methyl-thymidine (Amersham Buchler, Braunschweig, Germany) and harvested 16 hours later onto glassfiber filters. Incorporation of [<sup>3</sup>H]-thymidine was measured with a microplate counter (Wallac, Turku, Finland).

To assess the capability of infected DC to expand specific autologous cytotoxic T lymphocytes, DC were co-cultured with T cells (DC : T ratio of 1:20) in 24-well plates in RPMI supplemented with 10 % heat-inactivated human serum. After seven days of culture without addition of exogenous cytokines the specificity and functionality of CTL was evaluated by staining with tetramers (P.R. Dunbar *et al.*, Curr. Biol. 8, 413-416 (1998)) and by ELISPOT-assay.

9. Enzyme-linked immunospot assay (ELISPOT): The ability of the transduced DC to stimulate antigen-specific CTL clones (i.e. IFN- $\gamma$  production) and the specificity of expanded autologous T cells for defined peptides was measured on a single cell basis using the ELISPOT technique. Highly protein-binding nitrocellulose membrane-bottomed 96-well plates (MAHA S4510, Millipore, Eschborn, Germany) were precoated with 0.1  $\mu$ g/ml primary anti-IFN- $\gamma$  monoclonal antibody (1-D1k Mabtech, Stockholm, Sweden). After blocking with 5 % human serum  $0.5-1 \times 10^4$  antigen-specific T cells were added per well. Subsequently  $7.5 \times 10^4$  DC were carefully added. After 18 hours of culture at 37°C the plates were washed six times with 0.05 % Tween® in PBS and then incubated with biotinylated secondary anti-IFN- $\gamma$  monoclonal antibody (7-B6-1,

Mabtech, Stockholm, Sweden). Plates were washed again six times and stained with streptavidin/peroxidase (Vectastain Elite kit, Vector Laboratories, Berlingame, CA; USA) and 3-amino-9-ethylcarbazole (AEC, Sigma, Deisenhofen, Germany) as substrate. Spots were counted using a special computer assisted video imaging analysis system (Carl Zeiss Vision, Eching, Germany), using the KS ELISPOT software version 4.1.143.

10. Expression of the MAGE-3 tumor antigen using recombinant influenza viruses:

Since influenza viruses can infect dendritic cells (DC) in an abortive way and at the same time enhance their T cell stimulatory potential (M. Cella et al., J. Exp. Med. 189, 821-829 (1999)), influenza virus derived vectors should be well suited to express antigens in these cells, which subsequently will be efficiently presented to the Immune system. To introduce the tumor-associated antigen (TAA) MAGE-3 or, as a control, the green fluorescent protein (GFP) into influenza viruses, the RNA polymerase I (pol I) technique was applied (A. Zobel et al., Nucleic Acid Res. 21, 3607-3614 (1993)). The foreign gene is under the control of a high-capacity promoter (promoter 1104 having 3' modifications G3A, U5C and C8U and no 5' modification). To facilitate the detection of the protein, the MAGE-3 open reading frame was fused with the coding sequence for the AU-1 epitope tag. The coding sequences were inserted into the vector with the pol I promoter and viral packaging/promoter sequences (Fig.1). Due to the viral control sequences including the packaging signal, the pol I transcripts were incorporated into influenza virus particles when the transfected culture was inoculated with the avian influenza virus strain. Four to eight hours after infection, the cells started to stain green in UV light due to the expression of GFP (data not shown). The fraction of recombinants within the viral stock was determined by measuring their capacity to generate plaques and to express GFP in limiting dilution assays. Using the GFP recombinant virus as a model for the enrichment of the additional segment, we found that the heterologous segment is enriched from passage to passage. Starting from about 1-3 %, the content of recombinants in the viral stocks increased up to 30fold after two to three passages on MDCK cells (data not shown). This enrichment is due to the optimized viral promoter and packaging signal sequences present in the heterologous segment, which allow for a more efficient replication in comparison with the homologous influenza virus RNA segments (G. Neumann, G. Hobom, J. Gen. Virol. 76, 1709-1717 (1995)).

To check for the presence of MAGE-3 recombinants in viral stocks, MDCK cells were inoculated with virus and MAGE-3 transcripts were detected from total cellular RNA by RT-PCR (Fig. 2A). To verify the MAGE-3 expression on the protein level, total protein extracts from infected MDCK cells were prepared. The heterologous protein was detected in immunoblots. As shown in Fig.2B high amounts of heterologous MAGE-3 protein could be detected. The protein expression of GFP was high resulting in a similar band intensity as MAGE-3 (Fig.2B). The recombinant viruses expressing GFP or MAGE-3 were termed FPV-GFP and FPV-MAGE3-AU1, respectively.

11. Efficient transfer of MAGE-3 into mature human DC using recombinant influenza virus: To investigate whether heterologous genes can be transferred into human DC using recombinant Influenza viruses, mature DC were generated.

For this purpose CD14<sup>+</sup> monocytes were isolated from peripheral blood and treated with GM-CSF and IL-4 for six days; subsequently the cells were matured by the addition of IL-1 $\beta$ , IL-6, TNF- $\alpha$  and PGE<sub>2</sub>. These cells showed the typical phenotype of fully mature DC (see 6. above). To determine whether human monocyte-derived DC are accessible to gene transfer by recombinant influenza virus, the mature DC were infected with GFP encoding viruses at a MoI of 1. Microscopic examination revealed that almost all cells expressed the marker protein GFP (Fig. 3A). As expected the infection of these mature DC was abortive and did not result in cell lysis or production of progeny virus (data not shown). Quantitative analyses of green fluorescence using FACS showed that more than 90% of these DC were infected and expressed the heterologous protein (Fig. 3B). Maximal levels of GFP expression were detected within eight hours after infection. The protein was expressed for more than five days as was determined by daily examination of GFP fluorescence by microscopy. To verify the expression of the MAGE-3 gene, DC cultures were infected with the appropriate recombinant viral vector and in parallel with the GFP expressing virus. Protein extracts were subjected to immunoblot analyses. Both, the MAGE-3 gene and GFP gene were found to be expressed at high levels. For both proteins prominent specific bands of similar intensity could be demonstrated by using either an antibody binding the AU-1 tag of MAGE-3 or a GFP specific antiserum, respectively. (Fig. 4). Immature DC could be transduced with a

similar efficiency (data not shown). However in the immature cell population infection with a MoI of 1 did result in increased cytopathic effects.

12. Phenotypic characterization of influenza virus infected DC:

To investigate whether infection with recombinant influenza virus affects the phenotype of DC, typical cell surface markers were analysed by FACS 24 hours after infection with the GFP-coding recombinants. As shown in Fig. 5, the transduced DC produced high levels of the typical surface markers. About 100% of the GFP-positive cells expressed CD86, CD 80, HLA class II and more than 80% of those cells HLA class I molecules. Other characteristic cell surface markers such as CD25, CD40 and CD1a were also detected on GFP<sup>+</sup> DC. Comparison with uninfected DC did not reveal any major differences in any of these markers. A modest down-modulation of CD83 surface expression was the only deviation observed (data not shown). The same expression patterns were obtained for DC infected with FPV-MAGE3-AU1 or wild-type virus. These data indicate that the infection with recombinant Influenza virus and the high level expression of the heterologous antigens does not cause major phenotypic alterations in the infected DC .

13. T cell stimulation by Influenza virus transduced DC:

The crucial point for the application of DC in immunotherapy is their capacity to stimulate both CD4<sup>+</sup> helper as well as CD8<sup>+</sup> cytotoxic T cells (CTL). In order to address this issue, we firstly tested the ability of the transduced DC to induce T cell proliferation in allogeneic MLR. Therefore, DC were infected with one of the recombinant influenza virus strains or with FPV wild-type and co-cultured for four days with allogeneic CD4<sup>+</sup> T cells. After a 16 hours pulse, the incorporation of [<sup>3</sup>H]-thymidine was measured. As five individual experiments showed, all co-cultures resulted in a significant increase in [<sup>3</sup>H]-thymidine incorporation, even though 300 and more T cells per DC were present. This indicated that DC infected with FPV-GFP or with FPV-MAGE3-AU1 or with FPV wild-type have an unimpaired high T cell stimulatory capacity (Fig.6). Similar results were obtained with allogeneic CD8<sup>+</sup> T cells (data not shown). Secondly, In order to evaluate the capacity of influenza virus transduced DC to present viral antigen to CD8<sup>+</sup> CTL, transduced DC were co-cultured with an influenza virus matrix protein (IMP) specific CTL clone and the activation was determined by IFN- $\gamma$  ELISPOT (T. Hanke et al., vaccine 16, 426-435 (1998)). As shown in three individual experiments, influenza virus transduced DC were potent stimulators of this T cell clone,

indicating the presence of MHC-I/IMP complexes on its surface (Fig. 7). In contrast, uninfected DC with CTL or the CTL clone alone did not show any activation. Thirdly, we proved that these DC were capable to expand CD8<sup>+</sup> memory T cells in the absence of exogenously added cytokines; this is an unique property of DC. To this end, DC were infected with FPV-GFP or FPV-MAGE3-AU1. Uninfected DC and IMP peptide pulsed DC were prepared as negative and positive control, respectively. The DC were co-cultured with autologous CD8<sup>+</sup> T cells at a responder/stimulator ratio of 20:1. Specifically activated T cells were allowed to proliferate during a seven days culture period. Subsequently the number of IMP-specific CD8<sup>+</sup> cells were identified by double staining using monoclonal antibodies and tetrameric MHC-I/IMP complexes binding to specific T cell receptors (P.R. Dunbar et al., Curr. Biol. 8, 413-416 (1998)). As shown in Fig. 8A the infection with influenza virus recombinants resulted in an 11 to 18-fold increase of IMP specific CD8<sup>+</sup> positive T cells (for FPV-GFP: 0.49%, for FPV-MAGE: 0.32%; uninfected: 0.03%). Comparable numbers of specific CD8<sup>+</sup> T cells (0.57%) were obtained with IMP peptide pulsed DC. To assess whether the effector T cells expanded by the co-cultivation can be specifically activated, they were incubated with IMP peptide and analyzed for IFN- $\gamma$  production. As shown by ELISPOT assays, the number of individual IFN- $\gamma$  -producing T cells after co-culture with infected DC was about 30 times higher than that of the negative control (Fig. 8B). Next we investigated the ability of the FPV-MAGE3-AU1 transduced DC to generate MHC-I/MAGE-3 peptide complexes using a MAGE-3 specific CTL clone and ELISPOT as test system. As shown in Fig. 9, the transduced DC were able to stimulate IFN- $\gamma$  production from this CTL clone whereas the clone by itself did not spontaneously secrete IFN- $\gamma$ . Hence, TAA gene transfer by Influenza virus vectors into DC yields antigen presenting cells, which present the corresponding tumor antigen apart from vector antigen and which specifically stimulate T cells.

**CLAIMS**

1. A method for preparing patient derived immune cells capable of expression of one or more tumor-associated antigens (TAA) or virus-associated antigens (VAA), said method comprising
  - (a) preparing a recombinant influenza virus having at least one additional segment containing
    - (i) a nucleotide sequence coding for the TAA or VAA, and
    - (ii) terminal viral RNA sequences which have been modified by nucleotide substitutions in up to five positions, resulting in improved transcription rates of both the vRNA promoter as well as the cRNA promoter as present in the complementary sequences; and
  - (b) infecting patient derived immune cells with the recombinant influenza virus obtained in step (a).
2. The method of claim 1, wherein the at least one additional segment is a monocistronic viral RNA.
3. A method for preparing patient derived immune cells capable of expression of one or more tumor-associated antigens (TAA) or virus-associated antigens (VAA), said method comprising
  - (a) preparing an attenuated recombinant influenza virus containing a nucleotide sequence coding for the TAA or VAA, wherein said nucleotide sequence is incorporated into the influenza virus genome resulting in an attenuated phenotype; and
  - (b) infecting patient derived immune cells with the recombinant influenza virus obtained in step (a).
4. The method of claim 3, wherein the segment carrying the nucleotide sequence coding for the TAA or VAA contains terminal viral RNA sequences which have been modified by nucleotide substitutions in up to five positions, resulting in improved transcription rates of both the vRNA promoter as well as the cRNA promoter as constituted by the complementary sequences.
5. The method of claim 1, 2 or 4, wherein the 12 nucleotide conserved influenza 3' terminal sequence has been modified by replacement of one to three

nucleotides occurring in said sequence at positions 3, 5 and 8 relative to the 3' end by other nucleotides, and/or wherein the 13 nucleotide conserved influenza 5' terminal sequence has been modified by replacement of one or two nucleotides occurring in said sequence at positions 3 and 8 by other nucleotides.

5

6. The method of claim 5, wherein the replacements in the 3' terminal nucleotide sequence comprises

- (i) the modifications G3A and C8U, or G3C and C8G, and /or
- (ii) the modifications G3A, U5C and C8U, or G3C, U5C and C8G.

10

7. The method of claim 6, which comprises a 3' terminal nucleotide sequence of (5')-CCUGUUUCUACU-3' or (5')-CCUGUUUUUACU-3'.

15

8. The method according to any one of claims 5 to 7, wherein the 5' terminal nucleotide sequence comprises the modifications U3A and A8U resulting in a 5'-terminal sequence of 5'-AGAAGAAUCAAGG.

9. The method according to any one of claims 1 to 8, wherein the recombinant influenza virus is a recombinant influenza A virus.

20

10. The method according to any one of claims 1 to 9, wherein

(i) the TAA is selected from the class of cancer/testis antigens and preferably is MAGE-3, NY-ESO-1 or HOM-MEL-40;

(ii) the VAA is selected from a Herpes simplex virus immediate early or early protein, a human papilloma virus-antigen, preferably L<sub>1</sub>, E<sub>5</sub> or E<sub>6</sub>, a Human Immunodeficiency Virus protein, preferably tat, rev, nef or gag, or a Hepatitis C virus protein known to elicit T cell response in a mammal; or

(iii) the TAA or VAA is a fusion protein, preferably comprising entirely or partially the proteins listed in (i) or (ii) above; or

(iv) the encoded antigen is a microbial or parasitic protein known to induce cytotoxic T cell responses.

11. The method according to any one of claims 1 to 10, wherein the patient derived cells are

- (i) antigen presenting cells, preferably dendritic cells, and/or
- (ii) derived from a mammal, preferably a human being.

12. Patient derived immune cells, preferably dendritic cells expressing TAA or VAA which are obtainable according to the method of claims 1 to 11.

5 13. A pharmaceutical composition comprising patient derived immune cells of claim 12 or a mixture of different dendritic cells of claim 12, each expressing a different TAA or VAA.

10 14. Use of patient derived Immune cells of claim 12 for preparing a medicament for tumor therapy or antiviral therapy.

15 15. A method for the expression of tumor-associated antigens or virus associated antigens in patient-derived immune cells which comprises utilizing patient-derived immune cells of claim 12.

20 16. A method for treating tumors or virus infections in a mammal comprising administering the mammal patient derived Immune cells of claim 12.

Fig. 1 1/9

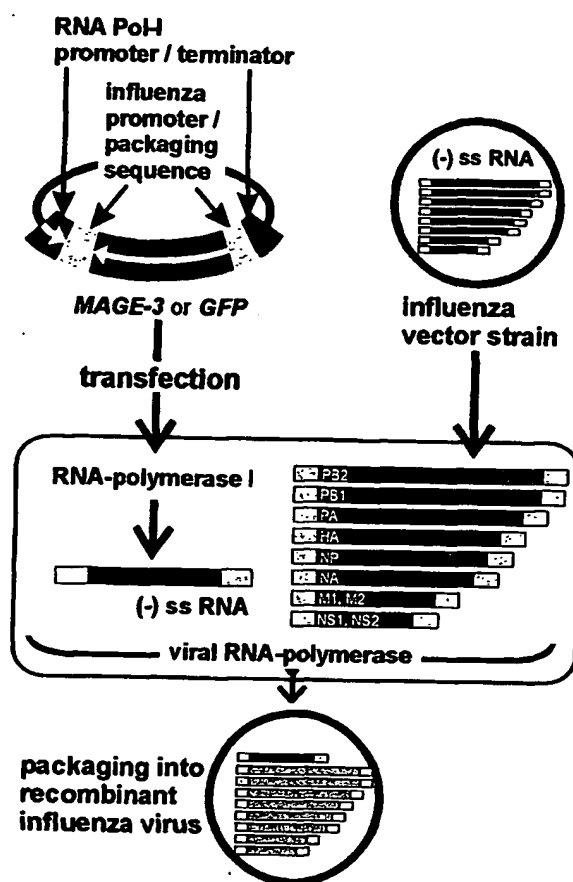
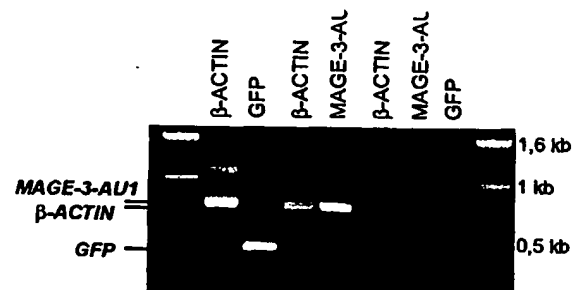
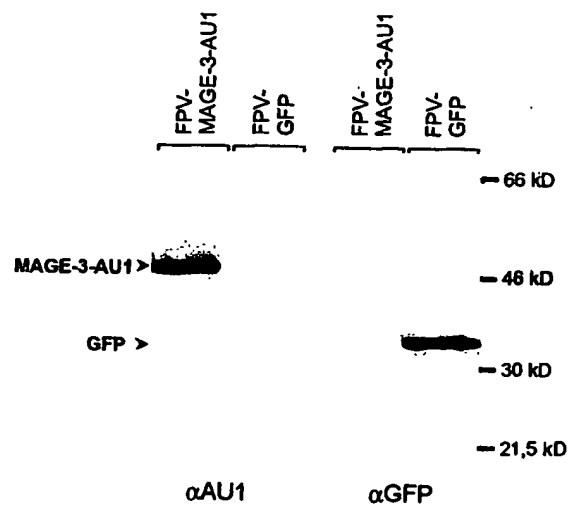


Fig. 2



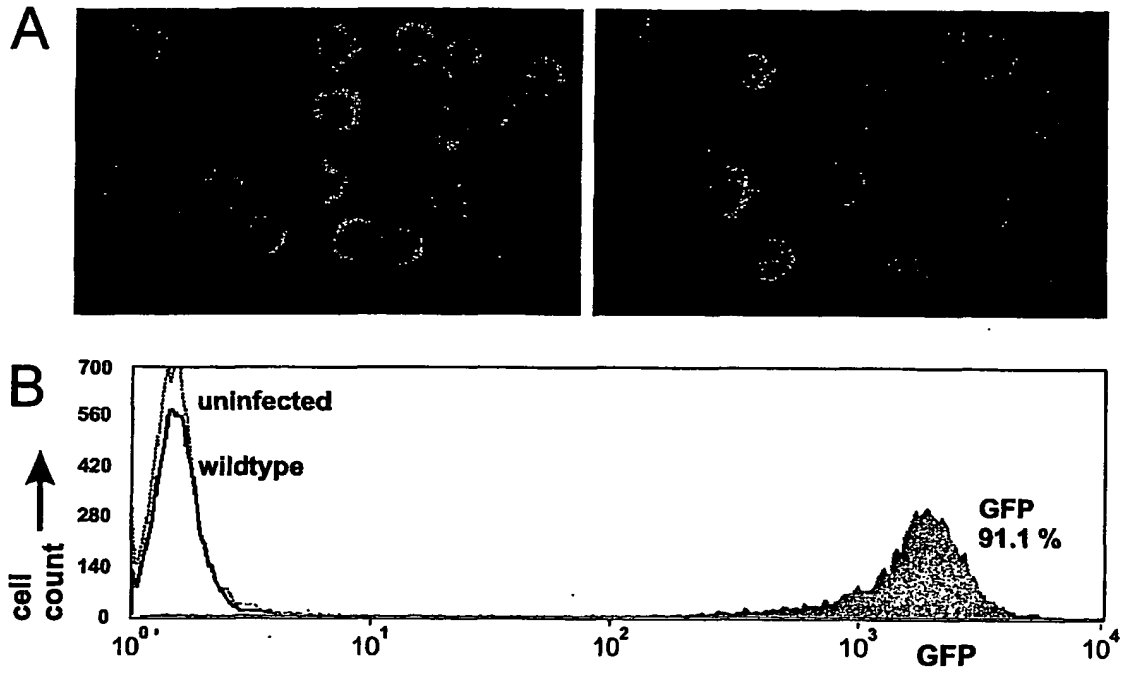
## A RT-PCR



## B Immunoblot

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Fig. 3



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Fig. 4

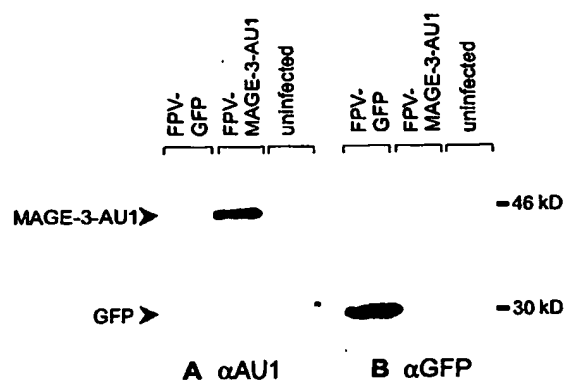


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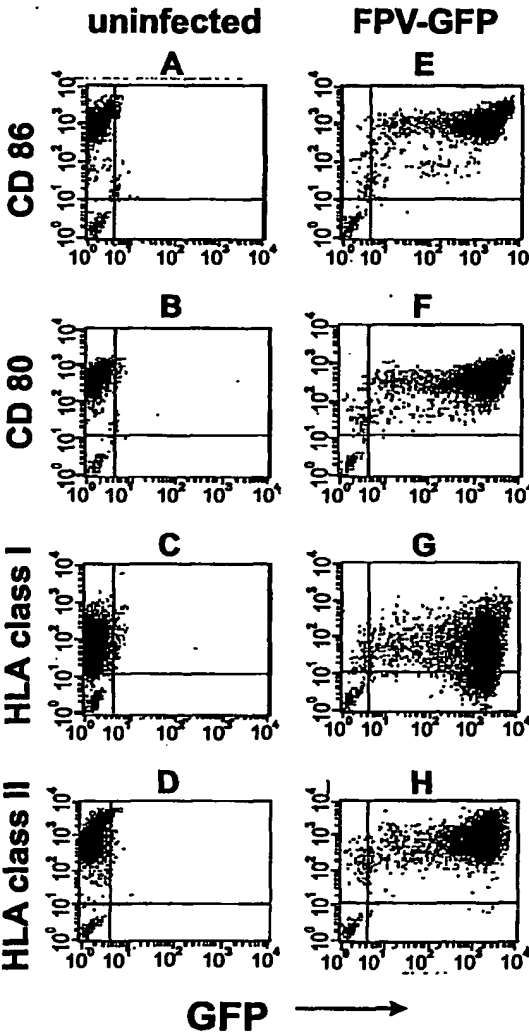
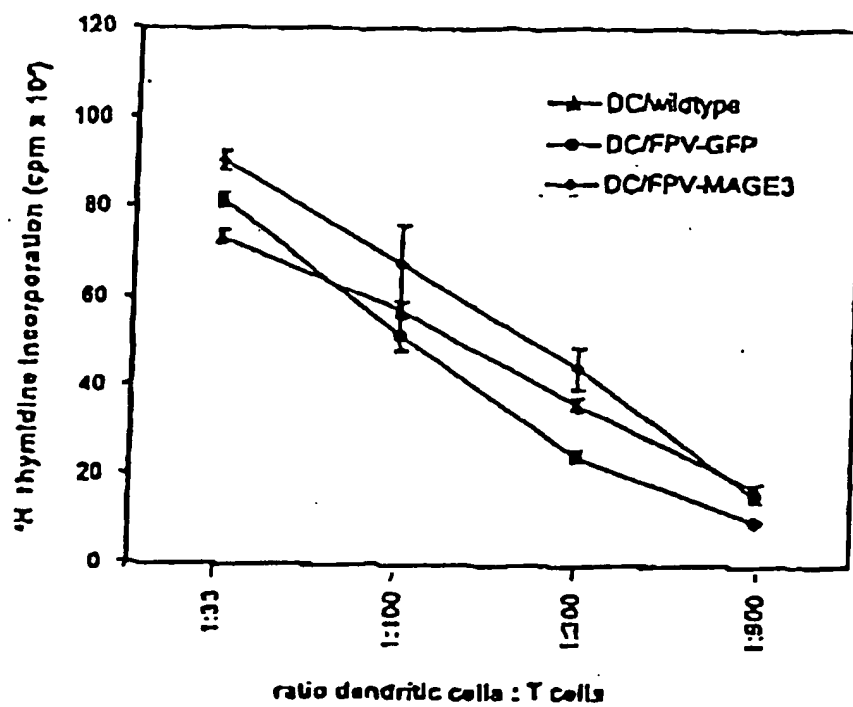


Fig. 6



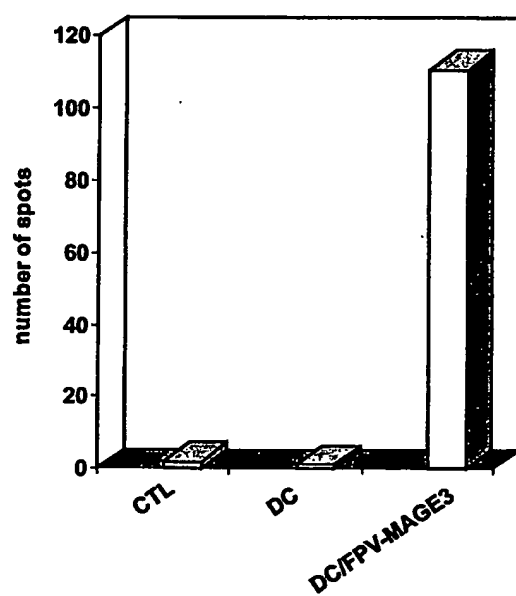


Fig. 8a

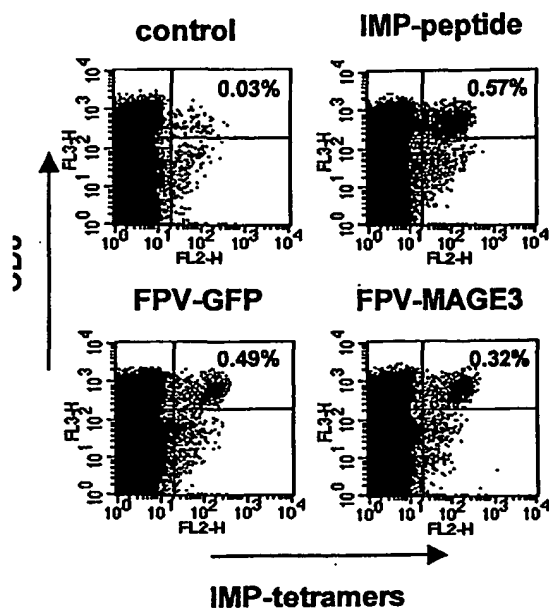


Fig. 8b

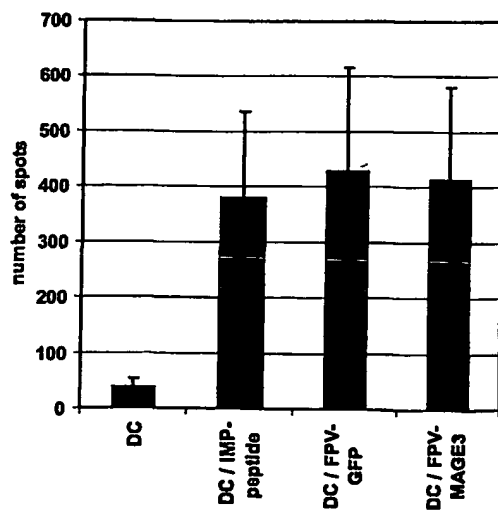
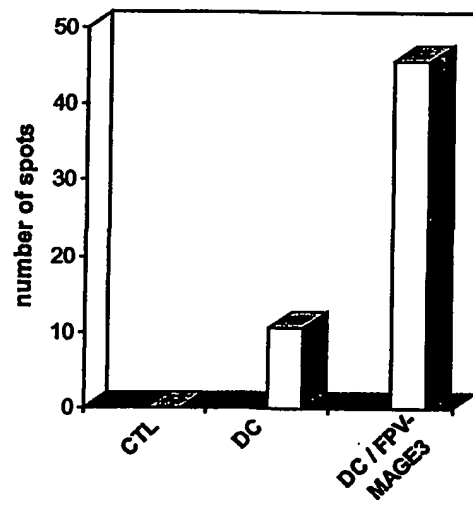


Fig. 9



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